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The Acidic Nature of the CcmG Redox-Active Center Is Important for Cytochrome *c* Maturation in *Escherichia coli*

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Cytochrome *c* biogenesis in *Escherichia coli* is a complex process requiring at least eight genes (*ccmABCDEFGH*). One of these genes, *ccmG*, encodes a thioredoxin-like protein with unusually specific redox activity. Here, we investigate the basis for CcmG function and demonstrate the importance of acidic residues surrounding the redox-active center.

Type *c* cytochromes are heme-binding proteins that function as electron transfer proteins in photosynthetic and/or respiratory chains. Unlike other classes of cytochromes, *c*-type cytochromes bind heme covalently (28). This binding requires the formation of two thioether bonds between the vinyl groups of heme and two cysteines in the conserved Cys-X-X-Cys-His motif of the apocytochrome. Three different posttranslational systems have evolved to facilitate cytochrome *c* formation in vivo (15). The most complicated of these systems, system I, is found in many gram-negative bacteria. For example, at least eight genes (*ccmABCDEFGH*) are essential for cytochrome *c* maturation in *Escherichia coli* (30). This is in stark contrast to the system of vertebrates and invertebrate mitochondria (system III), in which a single enzyme (cytochrome *c* heme lyase) facilitates the process (27). For system II, found in gram-positive bacteria, cyanobacteria, and chloroplasts, four accessory proteins have so far been identified (16).

One of the critical steps in the biogenesis of cytochrome *c* in *E. coli* is the ligation of heme to apocytochrome *c*. Because ligation occurs in the oxidizing environment of the periplasm, the heme-binding cysteines are likely to be oxidized by disulfide oxidases in this compartment. This idea is supported by the finding that *E. coli* strains deficient in the periplasmic protein oxidants DsbA and DsbB do not synthesize cytochrome *c* (22). Furthermore, recent evidence has shown that the Cys-X-X-Cys-His motif in apocytochrome *c* is capable of forming a disulfide bond and that this disulfide prevents the formation of mature cytochrome *c* in vitro (5). However, DsbA may not be involved directly in the maturation of cytochromes *c* (1, 6).

Several findings point to CcmG facilitating the reduction of apocytochrome *c* in vivo before heme attachment. Probably the most convincing evidence is that CcmG homologues contain a conserved Cys-X-X-Cys motif that is redox active (10) and that is required for its role in cytochrome *c* maturation in vivo (8). The Cys-X-X-Cys motif is characteristic of thioredoxin-like (TRX-like) proteins, of which thioredoxin (TRX) is the archetype. TRX maintains a reducing environment in the cytoplasm by reverting disulfide bonds to dithiols in cytoplasmic proteins (12). However, the redox activity of CcmG is different from that of TRX and TRX-like proteins in that it is highly specific and limited to cytochrome *c* maturation (10, 23). Furthermore, CcmG, unlike other TRX-like proteins, is not a catalyst of the insulin reduction assay (10). In addition, CcmG is reduced by the transmembrane electron transfer protein DsbD, supporting the notion that it plays a reducing role in cytochrome *c* maturation (14).

The crystal structure of a CcmG homologue from *Bradyrhizobium japonicum* was recently determined (7) and revealed the presence of a core TRX fold with several distinguishing features. We have previously shown that one of these features, an insert in the TRX fold, is required for CcmG function (7). Another distinguishing feature is the acidic nature of the CcmG redox-active center compared with those of other TRX-like proteins (7). Three conserved acidic residues (Asp97, Glu98, and Glu158 in *B. japonicum* CcmG) contribute to the negative charge. Here, we investigated the role of these acidic residues and other conserved features of CcmG in cytochrome *c* maturation by testing the ability of *E. coli* CcmG mutants to complement cytochrome *c* maturation in a Δ*ccmG E. coli* strain.

Asp107 and Asp129 in the central insert are not required for cytochrome *c* maturation. Asp107 and Asp129 of *E. coli* CcmG (Fig. 1) are surface exposed and highly conserved in CcmG homologues. Furthermore, both are part of the central insert in CcmG, which is required for cytochrome *c* maturation (7). To investigate a possible role for Asp107 and Asp129, each was mutated to alanine and the resulting *ccmG* mutant was used to transform a Δ*ccmG E. coli* strain containing a plasmid directing apocytochrome *c* to the periplasm (25). Cells expressing $CcmG_{Asp107Ala}$ or $CcmG_{Asp129Ala}$ were grown under anaerobic conditions to induce the expression of chromosomal cytochrome *c* maturation genes (*ccmABCDEFH*). Periplasmic proteins were isolated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and holocytochrome *c* was detected by heme staining of the electrophoresed proteins

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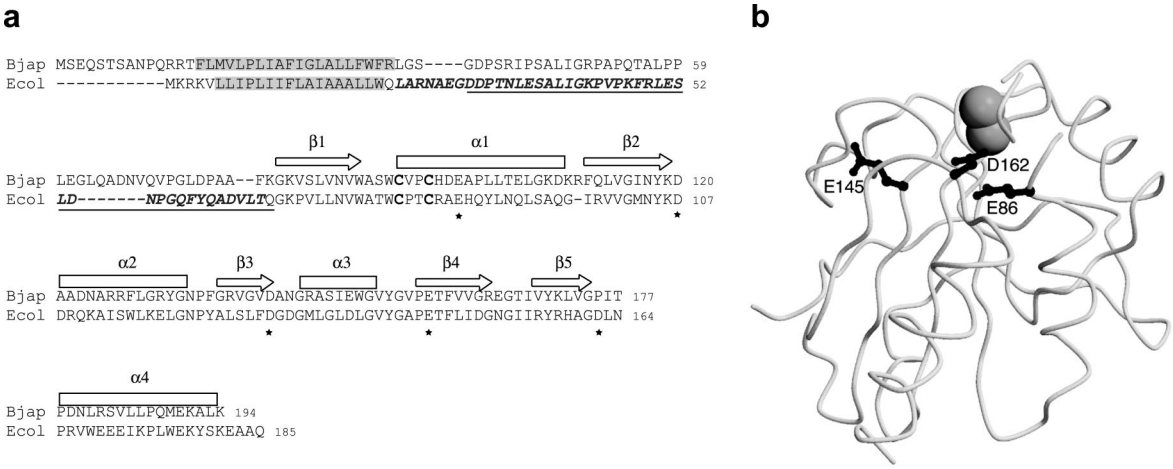

FIG. 1. CcmG structure and sequence. (a) Sequence alignment of *E. coli* and *B. japonicum* CcmGs. Residues mutated in *E. coli* CcmG are marked by asterisks. The residues deleted to create $CcmG\Delta_{Leu24\text{-}Thr66}$ are italicized, and the residues deleted to create $CcmG\Delta_{Asp31\text{-}Gln67}$ are italicized and underlined. The redox-active cysteines are shown in boldface type. Secondary-structure elements based on the *B. japonicum* structure are shown, and the predicted transmembrane region is boxed in gray. Sequences are from the Swissprot database. (b) Cartoon showing the structure of CcmG and the positions of the three acidic residues identified as important for function. The redox-active center is indicated by spheres for the sulfur atoms of the cysteines.

(26). Surprisingly, despite their prime positions and conservation in CcmG homologues, neither Asp107 nor Asp129 was required for CcmG function. Rather, both $CcmG_{Asp107Ala}$ (Fig. 2A, lane 1) and $CcmG_{Asp129Ala}$ (Fig. 2A, lane 2) were able to complement cytochrome *c* maturation to wild-type levels in a Δ*ccmG E. coli* strain.

The acidic nature of the redox-active center plays a role in cytochrome *c* maturation. The redox-active center of CcmG is relatively acidic (7) compared to other TRX-like oxidoreductases, including TRX (13), DsbA (20), DsbC (21), and the closest structural relative to CcmG, TlpA (2). Three acidic residues in CcmG, of which two are exposed to solvent (Asp97 and Glu158 in *B. japonicum* CcmG) and one is partly buried (Glu98 in *B. japonicum* CcmG), contribute to the acidic redox-active center. Glu98 and Glu158 are conserved in all CcmG homologues identified to date. Asp97 is conserved in three CcmG homologues (*B. japonicum*, *Rhizobium leguminosarum*, and *Haemophilus influenzae* homologue 2), and a nearby residue is often acidic in three other CcmG homologues (Asp162 in *E. coli*, Asp157 in *Pseudomonas fluorescens,* and Asp160 in *H. influenzae* homologue 1). These three acidic residues are all within 5 to 8 Å of the redox-active center (Fig. 1b). The finding that these three residues are conserved in CcmG but not in other TRX-like proteins suggests that the acidic nature of the CcmG redox-active center may be important for cytochrome *c* maturation. A functional role for these three residues in *E. coli* CcmG (Glu86, Glu145, and Asp162) (Fig. 1) was investigated by testing the ability of the respective single CcmG mutants (Glu86Ala, Glu145Ala, and Asp162Ala) to complement cytochrome *c* maturation in a Δ*ccmG E. coli* strain. However, all three single mutants complemented cytochrome *c* to wild-type levels in the Δ*ccmG E. coli* strain (Fig. 2A, lanes 3 to 5).

To further investigate a functional role for the three acidic residues near the redox-active center of CcmG, a CcmG double mutant ($CcmG_{Glu86Ala/Glu145Ala}$) and a CcmG triple mutant ($CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$) were made and characterized. Two of the three acidic residues involved are conserved in all CcmG homologues identified to date. These two residues were simultaneously mutated to alanines to create a CcmG double mutant. Cells expressing $CcmG_{Glu86Ala/Glu145Ala}$ produced lower levels of holocytochrome *c* (Fig. 2B, lane 1) than cells expressing wild-type CcmG (Fig. 2B, lane 3). This result supports the pro-

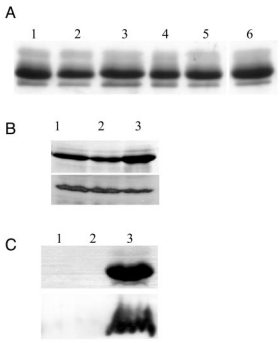

FIG. 2. Characterization of CcmG mutants. (A) Heme stain of cytochromes produced by CcmG single mutants (30 μg of periplasmic protein). Lane 1, $CcmG_{Asp107Ala}$; lane 2, $CcmG_{Asp129Ala}$; lane 3, $CcmG_{Glu86Ala}$; lane 4, $CcmG_{Glu145Ala}$; lane 5, $CcmG_{Asp162Ala}$; lane 6, $CcmG_{wild\text{-}type}$. (B) Characterization of CcmG double ($CcmG_{Glu86Ala/Glu145Ala}$) and CcmG triple ($CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$) mutants. Heme stain of 100 μg of periplasmic protein per lane (upper panel) and Western blot of whole-cell extracts obtained by trichloroacetic acid (TCA) precipitation of 0.5 optical density unit (at 600 nm) of cells per lane (lower panel) by using an antiserum against the CcmG peptide Asn104-Glu118. Lane 1, $CcmG_{Glu86Ala/Glu145Ala}$; lane 2, $CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$; lane 3, CcmG wild type. (C) Heme stain of CcmG N-terminal deletions; heme stain of 50 μg of periplasmic protein (upper panel) and Western blot with anti-His antibody of whole-cell extracts obtained by TCA precipitation of 1.5 optical density units (at 600 nm) of cells per lane (lower panel). Lane 1, $CcmG\Delta_{Leu24\text{-}Thr66}$; lane 2, $CcmG_{Asp31\text{-}Gln67}$; lane 3, $CcmG_{His}$.

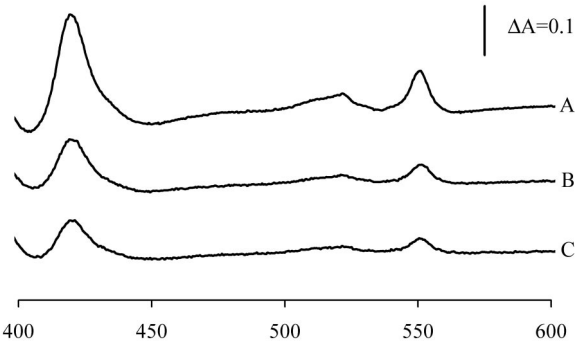

FIG. 3. Absorption difference spectra of *c*-type cytochromes. A Δ*ccmG* mutant expressing apocytochrome *c* was complemented with a plasmid expressing either wild-type CcmG (A), the double mutant $CcmG_{Glu86Ala/Glu145Ala}$ (B), or the triple mutant $CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$ (C). Periplasmic fractions were prepared from anaerobically grown cells, and protein levels were adjusted to 0.4 mg/ml. Dithionite-reduced spectra minus ammonium persulfate-oxidized spectra were recorded, and the $\Delta A_{551-536}$ value was used to determine the relative amounts of cytochrome *c*.

posal that the acidic nature of the redox-active center in CcmG is required for the specific function of CcmG in cytochrome *c* maturation. A triple mutant was also constructed by mutating to alanine each of the three acidic residues near the redox-active center in *E. coli* CcmG. Cells expressing the triple mutant ($CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$) (Fig. 2B, lane 2) produced lower levels of cytochrome *c* than cells expressing wild-type CcmG (Fig. 2B, lane 3).

Quantification of *c*-type cytochromes produced from cells carrying wild-type CcmG or the double-mutant or triple-mutant version was performed by absorption difference spectroscopy of periplasmic fractions (Fig. 3). The double mutant and the triple mutant produced 44 and 39%, respectively, of the type *c* cytochromes produced by the wild type (100%).

The N-terminal β-hairpin-like structure is associated with CcmG stability. The structure of CcmG includes an addition of ~30 residues to the TRX fold at the N terminus (7) that forms a β-hairpin like structure. The role of this addition to the TRX fold is unknown. In order to investigate a possible functional role, a *ccmG* deletion mutant was designed to link the membrane anchor in *E. coli* CcmG (Met1 to Trp22) (10) directly to the first strand of the TRX fold in CcmG by removing residues 24 to 66 ($CcmG\Delta_{Leu24-Thr66}$) (Fig. 1). This variant was unable to complement cytochrome *c* maturation in the Δ*ccmG E. coli* strain (Fig. 2C, upper panel, lane 1). The stability of $CcmG\Delta_{Leu24-Thr66}$ was investigated by Western blotting of total cell protein (with an anti-His tag antibody), which failed to detect protein (Fig. 2C, lower panel, lane 1), suggesting that the β-hairpin may play a role in stabilizing the protein. A second deletion that removed a shorter section of the N terminal region, leaving additional residues to connect the membrane anchor with the TRX fold of CcmG, was constructed. This variant of ($CcmG\Delta_{Asp31-Gln67}$) included seven more residues after the membrane anchor than the first deletion ($CcmG\Delta_{Leu24-Thr66}$) (Fig. 1). However $CcmG\Delta_{Asp31-Gln67}$, like $CcmG\Delta_{Leu24-Thr66}$, did not complement cytochrome *c* maturation in Δ*ccmG E. coli* (Fig. 2C, upper panel, lane 2) and also did not produce a stable product (Fig. 2C, lower panel, lane 2). Taken together, these results suggest that the β-hairpin-like structure at the N terminus of CcmG is required for stability. Perhaps this region is important for interacting with other Ccm proteins, an idea that is consistent with the proposal that Ccm proteins may associate at the membrane, forming a cytochrome *c* maturation complex (29).

Conclusions. Δ*ccmG E. coli* strains complemented with either $CcmG_{Glu86Ala/Glu145Ala}$ or $CcmG_{Glu86Ala/Glu145Ala/Asp162Ala}$ produced similar levels of cytochrome *c*. This result suggests that Asp162 is not as important for function as Glu86 or Glu145. This idea is consistent with the fact that Asp162 is not as highly conserved as Glu86 or Glu145. An alternative interpretation of these results is that the mutation of any two of the three acidic residues near the redox-active center is sufficient to create a mutant phenotype.

The double mutant ($CcmG_{Glu86Ala/Glu145Ala}$) produced lower levels of cytochrome *c* than wild-type CcmG did, indicating that the acidic residues are involved in CcmG function in *c*-type cytochrome maturation. Glu86 (Glu98 in *B. japonicum* CcmG) is located in a position in the TRX fold similar to that of Asp26 of *E. coli* TRX (13). Asp26 in TRX has been implicated in deprotonating the second cysteine in the Cys-X-X-Cys motif via a nearby water molecule (4, 17). Interestingly, *B. japonicum* TlpA, a periplasmic TRX-like protein that is required for the maturation of aa_3-type cytochromes (2, 19), contains an acidic residue (Glu78) that aligns with the equivalent residue in CcmG. Therefore, Glu86 in CcmG and Glu78 in TlpA are in a prime position to fulfill a role in catalysis similar to that of Asp26 in TRX.

The other highly conserved acidic residue, Glu145 (Glu158 in *B. japonicum* CcmG), follows the *cis*-Pro residue in the fingerprint motif of CcmGs (residues 139-Gly-Val-X-Gly-Ala/Val–*cis*-Pro–Glu–145). A *cis*-Pro in this position is conserved in TRX-like oxidoreductases and exposes the main chain oxygen of the preceding residue for interaction with other residues. The structurally equivalent regions in TRX (24) and DsbA (3) have been implicated in substrate binding. Based on these findings, Glu145 may also be involved in binding CcmG substrates. Possible substrates may be the electron donor DsbD or the electron acceptor apocytochrome *c*, though the latter interaction could be mediated by another redox-active Ccm protein, CcmH (9). The equivalent residue in TRX and TlpA is not acidic, suggesting that these proteins interact with different substrates.

The pK_a of the N-terminal cysteine at the redox-active center is used as a means of comparing biochemical and redox activities of TRX-like redox proteins. The pK_a of the thiol for *E. coli* CcmG (DsbE) is reported to be 6.8 (18), and that of the thiol for *B. japonicum* CcmG is expected to be similar, since the two proteins have similar reductant functions. By comparison, the pK_a of 5.0 is much lower for *Mycobacterium tuberculosis* DsbE (11). Although structurally very similar to CcmG, the oxidant properties of *M. tuberculosis* DsbE indicate that it is not involved in cytochrome *c* biogenesis (11). Our results support this notion, since *M. tuberculosis* DsbE lacks two of the three conserved acidic residues identified here as important for cytochrome *c* biogenesis. Furthermore, the fingerprint region indicative of CcmG function is not conserved in *M. tuberculosis* DsbE (Asn–Val–X–Trp–Gln–*cis*-Pro–Ala) (11). In this context, not only is the acidic residue Glu145 replaced by Ala but

the highly conserved hydrophobic residue that precedes *cis*-Pro in almost all TRX-like proteins is hydrophilic in *M. tuberculosis* DsbE. Hydrophilic residues at this position are also found in disulfide isomerases such as DsbC and DsbG, suggesting the possibility that *M. tuberculosis* DsbE may have isomerizing activity.

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